# Enhanced Neuropathogenicity of Avian Influenza A Virus by Passages through Air Sac and Brain of Chicks

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(Received 12 September 1996/Accepted 19 November 1996)

ABSTRACT. Three-day-old, specific-pathogen-free (SPF) chicks were inoculated with the strains of influenza A/whistling swan/Shimane/499/83 (H5N3) via the air sac route. The strains had been passaged through air sacs or air sacs and brains of SPF chicks. Two experiments were undertaken to examine the pathogenicity of these strains and the development of brain lesions based on time-interval changes. In experiment 1, original strain (4e) showed low pathogenicity with mild respiratory signs and zero mortality. Air sac passaged strains (18a and 24a) of 4e demonstrated mortalities of 50% and 67%, respectively, and inoculated chicks showed hemorrhages and necrotic lesions in major organs. Air sac-brain passaged strain (24a5b) of 4e produced 100% mortality and severe nervous signs. Severe circulatory disturbance with multiple foci of necrosis in major organs including the brain was found in chicks inoculated with 24a5b. The 24a5b was analogous to highly pathogenic avian influenza virus in regard to its pathogenicity to chicks. Hence, low pathogenic influenza virus (4e) gradually aggravated its pathogenicity to highly pathogenic virus (24a5b) by air sac and brain passages. In experiment 2, chicks were inoculated with 24a5b, and the earliest histological lesion was the enlargement of the vascular endothelial cells at 18 hr post-inoculation (PI) followed by necrotizing encephalitis at 24 to 48 hr PI. Immunohistological staining revealed avian influenza virus antigen initially in the vascular endothelial cells and then in the astrocytes, neurons and ependyma. — KEY WORDS: avian influenza virus, chick, neuropathogenicity, passaged-strain.

*J. Vet. Med. Sci.* 59(3): 143–148, 1997

Subtypes H5 and H7 of type A influenza virus (AIV) have the possibility to mutate into highly virulent AIV [7, 17]. This was illustrated by the single point mutation of the hemagglutinin (HA) gene of low pathogenic influenza virus (H5N2) that caused epidemic in the American poultry farms in 1983 [2, 11, 12, 26]. Several studies showed the recovery of highly pathogenic strain after *in vitro* passages of low pathogenic isolates [13, 16, 19]. There have been no published reports describing *in vivo* passages of low pathogenic isolates that yielded highly pathogenic strain.

In our previous report [24], low virulent (ICPI 0.20 to 0.40) AIV caused pancreatic atrophy and poor growth of chicks after 10 times passages of the virus through the chicken air sacs. The strain aggravated in pathogenicity to chicks infected the brain and induced mild, nonsuppurative encephalitis. We continued *in vivo* (chicken air sac and brain) passages of the strain and, thereafter, produced the AIV of highly virulent or fowl plague type. The present paper describes the two experiments in which the four selected passaged strains were compared to clarify on how pathogenicity of the AIV was changed by the passages (Experiment 1), and early pathological events in the development of brain lesions were investigated (Experiment 2).

## MATERIALS AND METHODS

*Virus*: The original isolate was influenza A/whistling swan/Shimane/499/83 (H5N3), and the strain was of low pathogenicity with ICPI 0.20–0.40 [20, 21]. Four selected serial passaged virus strains of the prototype virus were used namely: 4e (original isolate; passaged in embryonated eggs four times); 18a (passaged in air sac of chicks 18 times); 24a (continuation of 18a); and 24a5b (passaged in the brain of chicks five times beginning from 24a).

*Experimental designs*: In experiment 1, ninety 3-day-old specific-pathogen-free chicks (SPF) were allotted to six groups: (1) 4e strain: 12 chicks; (2) 18a strain: 12 chicks; (3) 24a strain: 12 chicks; (4) 24a5b strain: 38 chicks; (5) mock-inoculated control: 11 chicks; and (6) uninoculated control: 5 chicks. A volume of 0.2 m*l* of each of the virus strain under test ($10^{7.7}$–$10^{8.5}$ $EID_{50}$/0.2 m*l*) was inoculated into the caudal thoracic air sac of groups 1 to 4. In group 5, the same volume of sterile allantoic fluid was used as inoculum. Each group was housed separately and allowed free access to feed and water. Clinical signs were monitored twice daily for 7 days. Three chicks from 4e, 18a, and 24a were planned to be sacrificed at 1, 3, 5 and 7 days post-inoculation (PI). However, many chicks unexpectedly died or were moribund in 18a, 24a and 24a5b groups. Numbers of chicks necropsied at each day PI are collectively shown in Table 1. At necropsy, tissues from major organs of all chicks were collected for histopathology and immunohistochemistry.

In experiment 2, forty-three 3-day-old SPF chicks were allotted to 3 groups: (1) 24a5b strain: 26 chicks; (2) mock-inoculated control: 11 chicks; and (3) uninoculated control: 6 chicks. Group 1 was inoculated with 0.1 m*l* of the virus with a titer of $10^{6.2}$ $EID_{50}$/0.1 m*l* into the caudal thoracic air sac. The same volume was given in group 2 with sterile chorioallantoic fluid via the same route. At regular intervals of 6, 12, 18, 24, 27, 30, 36, 42 and 48 hr PI, 1 to 4 moribund or dead chicks of group 1 were necropsied. Two or one chicks from groups 2 or 3 were necropsied at the same time

Table 1. Number of chicks necropsied

| Groups | Days post-inoculation | | | | | | Total |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 7 | |
| 4e | 3* | 0 | 3 | 0 | 3 | 3 | 12 |
| 18a | 3 | 0 | 4 | 2 | 3 | 0 | 12 |
| 24a | 3 | 1 | 6 | 0 | 2 | 0 | 12 |
| 24a5b | 12 | 26 | 0 | 0 | 0 | 0 | 38 |
| Mock-inoculated | 3 | 3 | 2 | 0 | 2 | 1 | 11 |
| Uninoculated | 1 | 1 | 1 | 0 | 1 | 1 | 5 |

*: Total number of chicks necropsied.

as group 1. The brain and spinal cord (CNS) from all chicks were collected for histopathology and immunohistochemistry. In addition, pieces of the brain were processed for electron microscopy.

*Histopathology and immunohistochemistry*: Tissue samples collected were fixed in 2% periodate-lysin-paraformaldehyde (PLP) at 4°C for 24 hr. Tissues were routinely processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). For immunohistochemistry, duplicate paraffin-embedded sections were placed on 0.4% neoprene coated glass slides, deparaffinized, digested with trypsin and blocked non-specific reactions with normal swine serum. The sections were incubated overnight with primary antibody (rabbit anti-strain 499 hyperimmune serum at a 1:5,000 dilution) at 4°C after washing in phosphate buffered saline (PBS). The sections were incubated with biotinylated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) at room temperature for 1 hr. Then, they were washed in PBS and specific reaction was visualized with diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin and examined under light microscope.

*Ultrastructure*: Small tissue pieces of the brain from six chicks inoculated with 24a5b strain and died or killed at 24 and 36 hr PI were fixed in 2% PLP for 1 hr, cut into 1 $mm^3$ cubes, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Quetol 812 resin. Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a JEM-100 CX II electron microscope.

## RESULTS

*Experiment 1*

*Clinical findings*: In 4e group, infected chicks showed temporary sneezing and diarrhea. No deaths were recorded during the experimental period. In 18a and 24a groups, infected chicks demonstrated severe clinical signs of respiratory, digestive and nervous systems such as diarrhea, dehydration, ruffled feathers, mouth breathing, cyanotic wattles and comb, torticollis, ataxia, paralyzed wings and legs in several chicks. Within 5 days, mortalities of 18a and 24a groups reached 50% and 67%, respectively. In 24a5b group, infected chicks appeared cyanotic and dyspneic at 16 hr PI. Majority of the chicks developed nervous signs. Within 28 hr PI, 95% of the chicks died and 5% were moribund. Control chicks remained healthy throughout the experiment.

*Macroscopical findings*: In 4e group, a few chicks showed an opacity of air sacs, mild congestive edema and partial consolidation of the lungs at 3 days PI. Many chicks of 18a and 24a groups showed mild to moderate congestive edema of the lungs, yellowish-white foci in the liver, discoloration of the kidneys, and swelling or atrophy of the pancreas from 1 day PI. Hemorrhagic foci were found in the liver, kidneys, lungs, pancreas and bursa of Fabricius in some chicks of these groups. Chicks in 24a5b groups died or were moribund within 2 days PI invariably showed severe congestive edema of the lungs.

*Histopathology*: Histological changes were found mainly in the vascular endothelial cells, respiratory tracts, brain, lymphoid tissues, pancreas, liver, heart and kidneys. These changes were aggravated with the advancement of the viral passages (Table 2). Immunohistochemically, virus antigen was prominent in the systemic vascular endothelial cells from the early stages of infection and then in the degenerative and necrotic lesions of major organs with intense staining as the disease progressed. Control groups developed no histological changes nor positive immunohistochemical reactions in any of the examined sections.

Table 2. Histological changes of chicks inoculated with 4 passaged strains of type A influenza virus

| Lesion | Virus strains | | | |
|---|---|---|---|---|
| | 4e (n=12) | 18a (n=12) | 24a (n=12) | 24a5b (n=38) |
| Brain edema and necrosis | – | – | ± | ++ |
| Enlargement of vascular endothelial cells | ± | ± | + | + |
| Congestive edema of the lungs | ± | + | ++ | +++ |
| Pneumonia and air sacculitis | ± | ++ | ++ | ++ |
| Hepatocytic necrosis and hemorrhage | – | ++ | ++ | + |
| Nephritis | ± | + | + | ± |
| Necrotic pancreatitis | ± | ++ | ++ | ++ |
| Necrosis of lymphoid tissues | – | ± | + | +++ |

–: No lesion, ±: Very mild, +: Mild, ++: Moderate, +++: Severe, n: Number of chicks.

*4e*: There were occasional lymphocytic infiltration in the liver and pancreas, and very mild pneumonia and air sacculitis with infiltration of heterophils at 1, 3 and 5 days PI. On 7 days PI, two and one chicks had necrotizing nephritis and pancreatitis, respectively. Some degenerated alveolar epithelial and inflammatory cells of the lungs reacted positively for viral antigen in one to all of the three chicks at each day PI. From 3 days PI, weak to moderate positive reaction for vial antigen was found in vascular endothelial cells, myofibers of the heart, parenchymal cells of kidneys and pancreas, and necrotic lymphocytes of lymphoid organs in one to all of the three chicks. There were no histological changes nor viral antigen in the CNS.

*18a*: Moderate to severe pneumonia and air sacculitis were found in all chicks. From 3 days PI, many chicks showed congestive edema of the lungs, necrotic foci in the pancreas, kidneys and liver. Mild enlargement of vascular endothelial cells and lymphocytic necrosis in lymphoid organs appeared from 3 days PI in some chicks. CNS was unaffected. Immunohistochemistry demonstrated viral antigens in the lungs and vascular endothelial cells of many organs, hepatocytes, urinary tubular epithelial cells, lymphocytes and RES cells in lymphoid organs from 1 day PI, mucosal epithelial cells of the glandular stomach, smooth muscle cells of the digestive tracts and skeletal muscles of the thigh from 2 days PI, and cardiac muscle fibers from 3 days PI. Seven of nine chicks sacrificed between 3 to 5 days PI showed the antigen in a small number of astrocytes of the brain.

*24a*: Histological changes and distribution of viral antigen were similar to 18a, but they were more intense and widely distributed in 24a group. In addition to the histological changes described in 18a group, focal myocardial necrosis appeared from 3 days PI. CNS was unremarkable except one chick in which small necrotic focus was found in the cerebellum at 5 days PI. Viral antigen was found in many organs and tissues as described in 18a group for 1 or 3 days PI. The antigen in the brain appeared in nerve cells as well as vascular endothelial cells and astrocytes from 3 days PI.

*24a5b*: Edema and focal necrosis were found throughout the brain of all chicks at 1 and 2 days PI. The necrotic lesions were accompanied by necrosis of astrocytes and nerve cells, and perivascular vacuolation of neuropil. Histological changes of other organs were more intense in comparison with 18a and 24a groups. Viral antigen was detected from 1 day PI in many organs and the intensity of positive reaction was increased at 2 days PI. In the brain, positive granules appeared in the vascular endothelial cells, astrocytes, nerve cells, ependymocytes and neuropil. The viral antigen was also found in ganglion cells of Auerbach's plexus, smooth muscle cells of digestive tracts and skeletal muscle cells of the thigh.

*Experiment 2*

*Clinical findings*: Chicks inoculated with 24a5b strain were depressed from 12 hr PI and showed an unsteady gait, cyanosis of the comb, wattle and legs. Diarrhea, dehydration, dyspnea, torticollis, tremor to paresis of the wings and legs appeared from 24 hr PI in all chicks. Sixty-two percent of the chicks died and the remaining chicks were killed at moribund condition before 48 hr PI. Control chicks showed no abnormality throughout the experiment.

*Macroscopical findings*: Congestive edema of the lungs was found in all chicks. Fourteen of sixteen chicks necropsied between 24 and 48 hr PI showed malacic foci in the brain. There were no macroscopical abnormalities in control birds.

*Histopathology*

*6–12 hr PI*: There were neither lesions nor viral antigen in CNS of all chicks examined.

*18 hr PI*: Blood vessels contained enlarged endothelial cells in the brain, spinal cord, meninges and choroid plexus. Viral antigen was detected in the vascular endothelial cells, a few astrocytes and ependymocytes (Table 3).

*24 hr PI*: In addition to the enlargement of vascular endothelial cells, edema, focal hemorrhages, swelling of astrocytes, dilatation of perivascular and pericellular spaces throughout CNS, and tiny foci of necrosis in the cerebrum (Fig. 1) were also present. Viral antigen was found in nerve cells, astrocytes and ependymocytes as well as in the vascular endothelial cells of CNS (Fig. 2).

*27-48 hr PI*: In addition to the histological changes found at 18 and 24 hr PI, tiny foci of necrosis appeared throughout CNS and persisted till the termination of the experimental period. Positive reaction for viral antigen increased in degenerated or intact astrocytes and nerve cells of CNS from 30 or 48 hr PI (Fig. 3), whereas the antigen in vascular endothelial cells decreased from 30 hr PI (Fig. 4).

CNS of control chicks had no histological lesions and viral antigen.

*Ultrastructure*: At 24 and 36 hr PI, capillary endothelial cells of the brain showed swelling of nucleus and mitochondria, dilatation of endoplasmic reticulum, and

Table 3. Detection of viral antigen in specific cells of the brain

| | Hours post-inoculation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 6 (n=3) | 12 (n=3) | 18 (n=4) | 24 (n=2) | 27 (n=3) | 30 (n=3) | 36 (n=4) | 42 (n=3) | 48 (n=1) |
| Vascular endothelial cells | – | – | +++ | +++ | +++ | ++ | ++ | ++ | + |
| Astrocytes | – | – | + | ++ | + | ++ | ++ | +++ | ++ |
| Nerve cells | – | – | – | ± | – | + | + | + | ++ |
| Ependymocytes | – | – | ± | ± | ± | + | + | + | ± |

–: Nil, ±: Very mild, +: Mild, ++: Moderate, +++: Marked, n: Number of chicks.

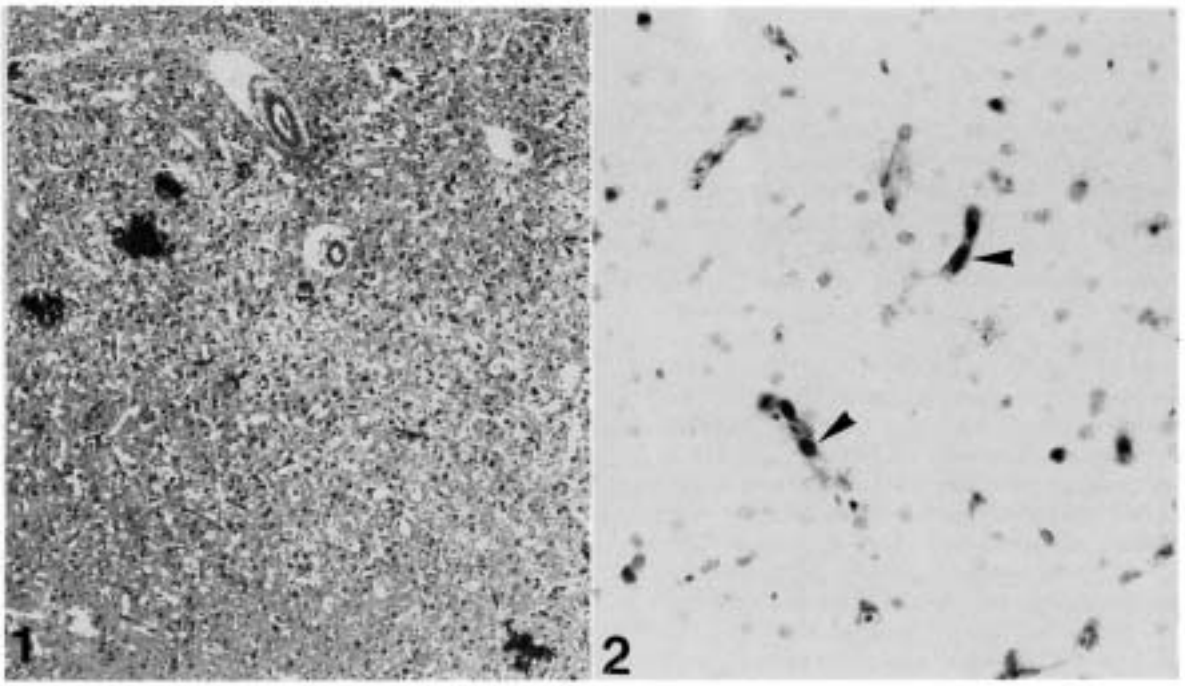


Fig. 1. 24a5b strain. Necrotic foci in the cerebrum with dilatation of perivascular and pericellular spaces at 24 hr PI. HE. × 75.
Fig. 2. 24a5b strain. Positive immunostaining of vascular endothelial cells (arrowheads) in the cerebrum at 24 hr PI. LSAB. × 190.

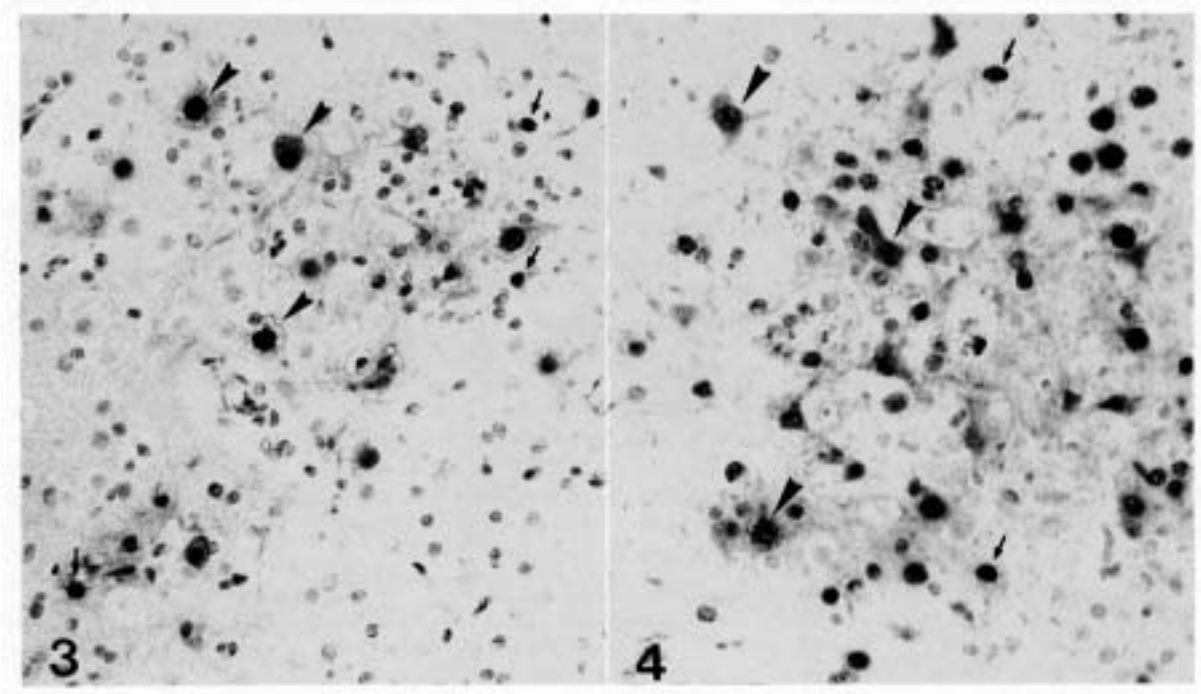


Fig. 3. 24a5b strain. Moderate amount of immuno-positive granules in the astrocytes (arrows) and nerve cells (arrowheads) of the cerebrum at 36 hr PI. LSAB. × 190.
Fig. 4. 24a5b strain. Large amount of immuno-positive granules in the astrocytes (arrows) and nerve cells (arrowheads) of the cerebrum at 42 hr PI. LSAB. × 190.

fragmentation of basement membrane. Around the capillaries, cytoplasmic processes of astrocytes and nerve cells were swollen. Budding of rod-shaped particles measuring 80 × 100–620 nm with 10 nm-thick envelope, was infrequently found at the luminal surface of the degenerated capillary endothelial cells.

## DISCUSSION

In the present experiment, the original isolate (4e) was a low virulence virus (ICPI 0.20 to 0.40) and its pathogenicity was gradually increased by the serial passages through the air sac and brain of chicks. Mortalities of 4e, 10a [24], 18a, 24a and 24a5b were 0%, 50%, 67% and 100%, respectively. The pathogenicity to visceral organs such as the liver, kidneys, pancreas, and lungs was intensified by passages of the strains through the air sac. The brain-passaged strain (24a5b) caused severe necrosis in the brain and systemic circulatory disturbance which was manifested by cyanosis of the comb, wattle and legs, and congestive edema of the lungs. The brain-passaged strain produced plaques in trypsin-free chicken embryo fibroblast cultures, and also showed marked pathogenicity for 8-day-old chicks, with 100% (14/14) mortality within 42 hr PI (unpublished data). These findings suggest that the brain-passaged strain was a fowl plague type or highly pathogenic strain of type A influenza virus [1, 3, 7, 9, 14]. To the best of our knowledge, this may be the first report in which a low virulent type A influenza virus converted into highly pathogenic or fowl plague type virus after experimental *in vivo* passages in chicks.

There have been two possible explanations for the emergence of a highly pathogenic strain: mutation of a viral gene [11, 12] and selection of a highly pathogenic strain from a heterogeneous population (quasispecies) present in the original isolate [4, 5]. Genomic comparison of the 4e and 24a5b revealed multiple mutations in the HA gene and the description on the details of these mutations will appear elsewhere. Viral infection in the vascular endothelial cells and astrocytes of the brain was found in chicks inoculated with the strain 10a [24], but was not present in chicks inoculated with the original isolate. Incidence and quantity of viral antigen and the severity of brain lesions increased in chicks inoculated with 18a, 24a and 24a5b strains. The pathogenicity of 24a to the brain was particularly intensified after brain passages in chicks. These results suggest that the emergence of the highly pathogenic strain was induced by stepwise accumulation of multiple mutations of the virus genome [6, 8, 22] and selection of strain with increased yield for brain cells during serial *in vivo* passages.

Among the literatures on the pathogenesis of neurotropic influenza viruses, there has been discrepancy whether the virus infects the vascular endothelial cells before entering the brain parenchyma [10, 15, 23] or infects the astrocytes directly without affecting the endothelial cells [18]. In experiment 2, the 24a5b initially infected and propagated in the endothelial cells of the brain, then injured the blood-brain barrier and, finally, invaded the astrocytes and nerve cells. Viral antigen in the vascular endothelial cells of the brain was initially detected at 18 hr PI and decreased from 30 hr PI. In the previous two reports [18, 25], viral antigen in the brain was examined at 12-hr intervals and early brain lesions observed at 18 hr PI of our experiment might not possibly be encountered in the previous experiments. Although we examined the brain lesions of only one highly virulent type A influenza virus, it is suggested from the present results that neurotropic influenza viruses infect the vascular endothelial cells at the early stage of infection, and propagate in the astrocytes and nerve cells, after breaking through the blood-brain barrier.

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